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SOX9 directly regulates the type-II collagen gene

Donald M. Bell[1*], Keith K.H. Leung[1*], Susan C. Wheatley[2], Ling Jim Ng[1], Sheila Zhou[3], Kam Wing Ling[1], Mai Har Sham[1], Peter Koopman[2,4], Patrick P.L. Tam[3] & Kathryn S.E. Cheah[1]

Mutations in human *SOX9* are associated with campomelic dysplasia (CD), characterised by skeletal malformation and XY sex reversal[1–3]. During chondrogenesis in the mouse, *Sox9* is co-expressed with *Col2a1*, the gene encoding type-II collagen, the major cartilage matrix protein[4]. *Col2a1* is therefore a candidate regulatory target of SOX9. Regulatory sequences required for chondrocyte-specific expression of the type-II collagen gene have been localized to conserved sequences in the first intron in rats, mice and humans[5–8]. We show here that SOX9 protein binds specifically to sequences in the first intron of human *COL2A1*. Mutation of these sequences abolishes SOX9 binding and chondrocyte-specific expression of a *COL2A1*-driven reporter gene (*COL2A1-lacZ*) in transgenic mice. Furthermore, ectopic expression of *Sox9 trans*-activates both a *COL2A1*-driven reporter gene and the endogenous *Col2a1* gene in transgenic mice. These results demonstrate that *COL2A1* expression is directly regulated by SOX9 protein *in vivo* and implicate abnormal regulation of *COL2A1* during chondrogenesis as a cause of the skeletal abnormalities associated with campomelic dysplasia.

We have identified a 309-bp fragment within the first intron of human *COL2A1* which can direct expression of a reporter gene (*COL2A1-lacZ*) to chondrogenic sites in transgenic mice (K.K.H.L., P.P.L.T. and K.S.E.C., unpublished). This fragment contains sequences homologous to regulatory motifs in the mouse[7] and rat[5] type-II collagen genes shown to be required for chondrocyte-specific expression (referred to as COL2C1 and COL2C2 respectively; Fig. 1*a*). The COL2C1 and COL2C2 sequences are similar to AACAAT and AACAAAG, the binding sites for SRY and other SOX proteins[9–13].

We have demonstrated[4] that SOX9 can bind to AACAAT (denoted here as SRYC). Here, we tested whether SOX9 can bind to the SOX4/SOX18 binding site AACAAAG (SoCM)[11,12], or to the SOX/SRY-like binding motifs COL2C1 and COL2C2, in *COL2A1* (Fig. 1*a*). In electrophoretic mobility shift assays (EMSA) using SOX9 fusion protein, DNA/protein complexes of retarded mobility were observed for all three binding motifs (Fig. 1*b–d*). The DNA/protein interaction was SOX9-specific as the mobilities of these complexes were further retarded by a SOX9 antibody[14] and they were absent in control assays. This 'supershift' was abolished by incubating the binding reaction and antibody with the immunogenic SOX9 peptide prior to PAGE (Fig. 1*b–d*). SOX9 did not bind to mutated COL2C1 (C1M) and COL2C2 (C2M) sequences (Fig 1*c,d*), indicating that SOX9 binding to these motifs is sequence-specific. In competition experiments with COL2C2 as the labelled probe, SoCM, COL2C1

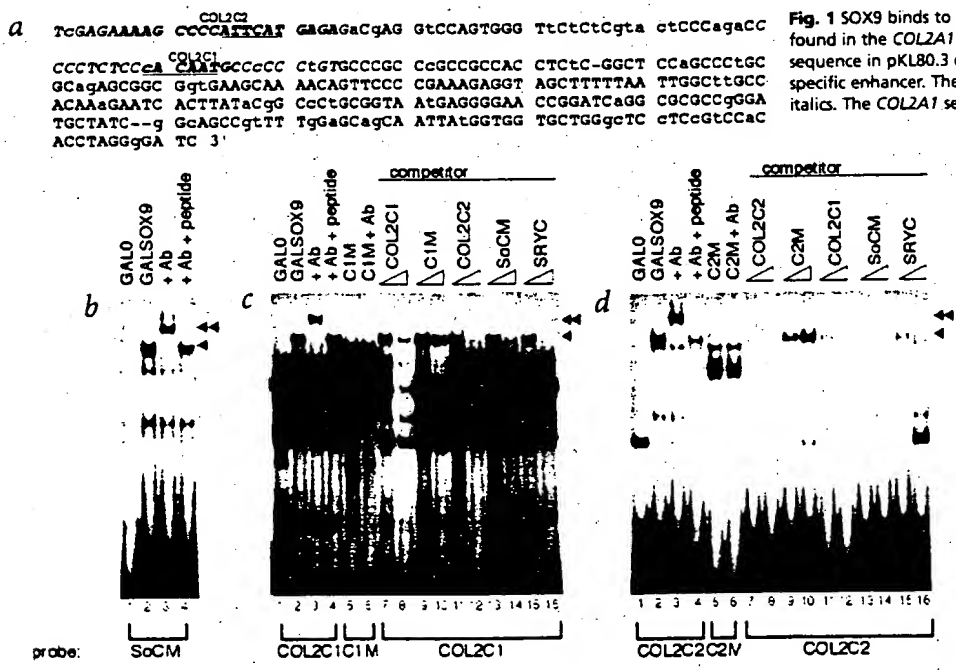

Fig. 1 SOX9 binds to SOX consensus motifs and novel motifs found in the *COL2A1* first intron. *a*, The 309-bp *COL2A1* intron sequence in pKL80.3 delineated as that containing a cartilage-specific enhancer. The sequences used for EMSA are shown in italics. The *COL2A1* sequences that are identical in rat and mouse are in uppercase and non-conserved sequences are in lowercase. The motif homologous to the rat decamer sequence[5], COL2C1, and the mouse 18-bp[7] COL2C2, are shown in italics and bold type. Sequences in COL2C1 and COL2C2 which were mutated (C1M and C2M) are underlined. ***b–d***, SOX9 binding to the different sequence motifs: *b*, SoCM; *c*, COL2C1 and C1M and *d*, COL2C2 and C2M. Oligonucleotides containing these consensus motifs (Methods) were used as EMSA probes. Track 1 in each panel shows complexes formed using a GAL0 extract (negative control); all other tracks contain GALSOX9 extract. Tracks 2 in *b–d* show a retarded band representing SOX9 bound to probe. The identity of the band was confirmed using an anti-SOX9 antibody (tracks 3, *b–d*) and antibody plus the immunogenic peptide which competes with SOX9 for binding to the antibody (tracks 4, *b–d*). In panels *c* and *d*, tracks 7–16 demonstrate competition between the labelled probe and excess cold oligonucleotides as marked on the figure. The arrowhead on the right hand side of each panel indicates the position of the SOX9 band. The double arrowhead indicates the position of the SOX9 antibody 'supershift'. Background DNA binding activity was found with COL2C1 but the same complexes were seen when using control GAL0 extracts, demonstrating that these were not SOX9-specific. In panels *c* and *d*, tracks 7–16 demonstrate competition between the labelled probe and 10- or 100-fold cold excess oligonucleotides as marked on the figure. The sloping triangle shown above each pair of competitions, represents x10 and x100 competitor. When labelled COL2C1 was used as a probe, competition with COL2C2 was greater than that with COL2C1 (*c*). However, with COL2C2 as the probe, the two oligonucleotides competed similarly (*d*) suggesting that SOX9 binds equally well to both *COL2A1* sequences. The high background DNA binding activity of COL2C1 (panel *c*) may have interfered, to some extent, with the competition analysis.

[1]*Department of Biochemistry, The University of Hong Kong, Sassoon Road, Hong Kong;* [2]*Centre for Molecular and Cellular Biology, and* [4]*Department of Anatomical Sciences, The University of Queensland, Brisbane 4072, Australia;* [3]*Children's Medical Research Institute, Locked Bag 23, Wentworthville 2145, Australia. Correspondence should be addressed to K.S.E.C. e-mail: hrmbdkc@hkumd1.hku.hk* *D.M.B. & K.K.H.L. contributed equally to this work.

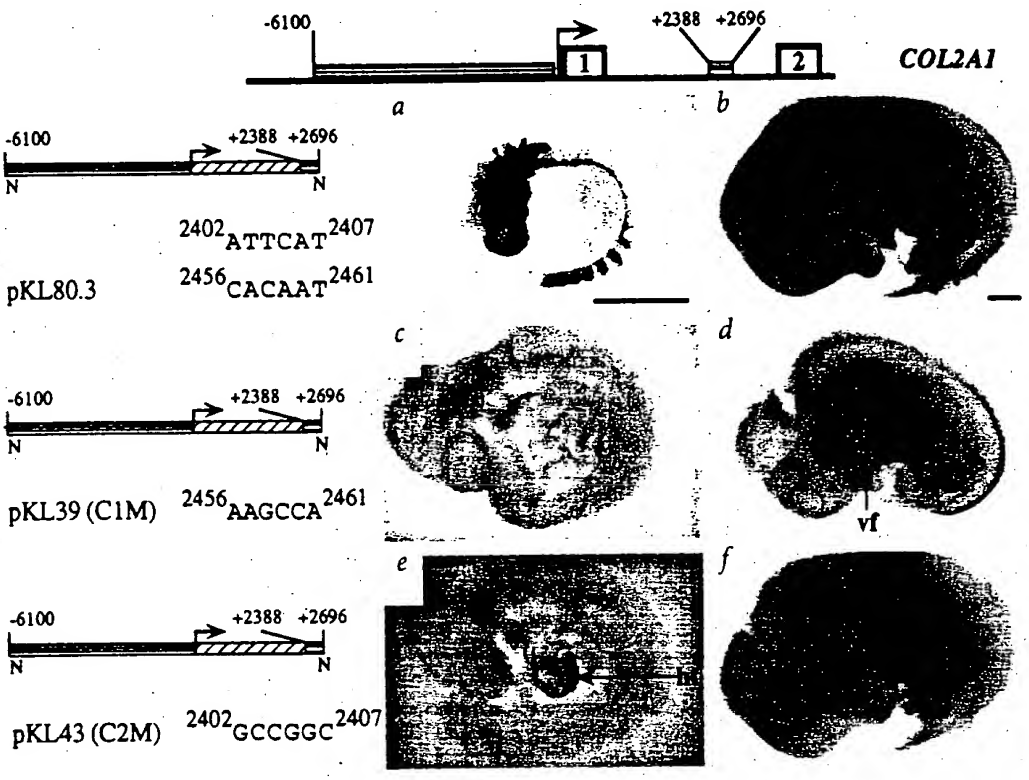

Fig. 2 Critical requirement of the SOX9 binding motifs COL2C1 and COL2C2 in the intron-1 enhancer for tissue-specific *COL2A1-lacZ* transgene expression. **a,b**, Expression of the *COL2A1-lacZ* (pKL80.3) construct in (*a*) the chondrogenic mesenchyme at 9.5 days and (*b*) the cartilaginous structures at 13.5 days. **c,d**, Effect of mutating COL2C1 (C1M mutation) in pKL80.3 in a 9.5-day embryo (*c*) and a 13.5-day fetus (*d*): 12 of 20 transgenic founders expressed *lacZ* but none showed the characteristic expression pattern of pKL80.3. **e,f**, Effect of mutating COL2C2 (C2M mutation) in pKL80.3: X-gal staining was seen in 17 out of 56 transgenic embryos (9.5–14.5 days) but *lacZ* expression was either weak, in subsets of chondrogenic tissues (6 founders), or, in certain sites, atypical of the *COL2A1* pattern (11 founders) as illustrated by representative 9.5-day (*e*) and 13.5-day (*f*) transgenic founders. Novel *COL2A1-lacZ* expression is found in the vibrissae follicle (vf) and the heart mesenchyme (ht). There is no significant difference in the rate of transgene integration and expression for the different constructs. Horizontal lined boxes, *COL2A1* sequence (coordinates shown are with respect to the transcription start site) included in the pKL80.3 construct; open boxes, exons; diagonally hatched boxes, the *lacZ* cassette including SV40 polyA tail; bent arrow, translation start site. Core sequences of the COL2C1 (+2456-2461) and COL2C2 (+2402-2407) motifs and their mutations (C1M, C2M) are shown below the line drawings. Bar = 1 mm.

and COL2C2 competed equally but to a greater degree than SRYC (Fig. 1*d*) suggesting that, of the consensus sequences tested, COL2C1, COL2C2 and SoCM are the preferred binding sites for SOX9.

To test whether the SOX9 binding sites COL2C1 and COL2C2 are required for chondrogenic expression of *COL2A1-lacZ in vivo*, we analysed the effect of mutating each of these sequences in transgenic mice. A *COL2A1-lacZ* reporter construct, pKL80.3, containing 6.1-kb 5' flanking DNA and 309 bp of first intron sequence (+2388 to +2696, Fig. 1*a*), including both SOX9 binding sites, was able to direct reporter gene expression to chondrogenic sites characteristic for type-II collagen (Figs 2, 3; K.K.H.L., P.P.L.T. and K.S.E.C., unpublished observations). Mutation of the COL2C1 (C1M) or COL2C2 (C2M) sequence in pKL80.3 (constructs pKL39, pKL43; Fig. 2) resulted in expression patterns which were totally different or weak or in subsets of chondrogenic tissues in 9.5–14.5 day embryos (Fig. 2*c–f* and data not shown). The same C2M mutation in a *COL2A1-lacZ* construct containing 2.1 kb of the first intron, completely abolished expression in 34 different transgenic founders (data not shown). The

Table 1 • Gene expression in *Hoxb2-lacZ*, *COL2A1-lacZ* single transgenic and *COL2A1-lacZ/Hoxb2-Sox9* double transgenic embryos

Transgenic Genotype (n)	Gene expressed	Expression site [n]									
		r3	hb r4	r5	ba1	ba2	ba3	nd	lm	som	nt
Hoxb2-lacZ(25)	*lacZ*	++[22]	+[22]	++[22]	+[11[c]]	+[22]	+[17]	-[25]	+[20]	+[18]	+[22]
COL2A1-lacZ(96[a])	*lacZ*	-	-	-	+	+	-	+	-	+	-
	Sox9 (3[d])	+[e]	+[e]	+[e]	+	+	-	+	-	+	+[f]
	Col2a1 (3[d])	+[e]	+[e]	+[e]	+	+	-	+	-	+	+[f]
COL2A1-lacZ(24)	*lacZ*	+[8]	+[19]	+[2]	+[21]	+[21]	+[18]	+[21]	+[21]	+[21]	-[24]
+ *Hoxb2-Sox9*	*Sox9* (3[b])	++[e]	++[e]	++[e]	+	+	+	+	+	+	+[f]
	Col2a1 (3[b])	+[e]	+[e]	+[e]	+	+	+	+	+	+	+[f]

Abbreviations: hb, hindbrain; r, rhombomere; ba, branchial arch; nd, notochord; lm, lateral mesoderm; som, somites; nt, neural tube; (n), number of different transgenic founder embryos analysed; [n], number of total founder embryos expressing lacZ at a particular site; [a]all transgenic embryos from pKL80.3 line; [b]number of double transgenic embryos analysed by *in situ* hybridisation; +, expressed; ++, upregulated expression; -, not expressed; [c]restricted to a few cells in the anterior part of ba1 whilst *COL2A1/LacZ* expression is throughout ba1; [d]*Sox9/Col2a1* expression pattern as described[4,29,30]; [e]Weak endogenous expression present4; [f]Weak endogenous expression present in an AP gradient[4]. In the hindbrain *trans*-activation of *COL2A1-lacZ* was graded and patchy and *trans*-activation in r4 appeared in 19 founders out of which nine expressed *lacZ* only in r4, eight in r3 and 4, and two in all three rhombomeres (r3,4,5).

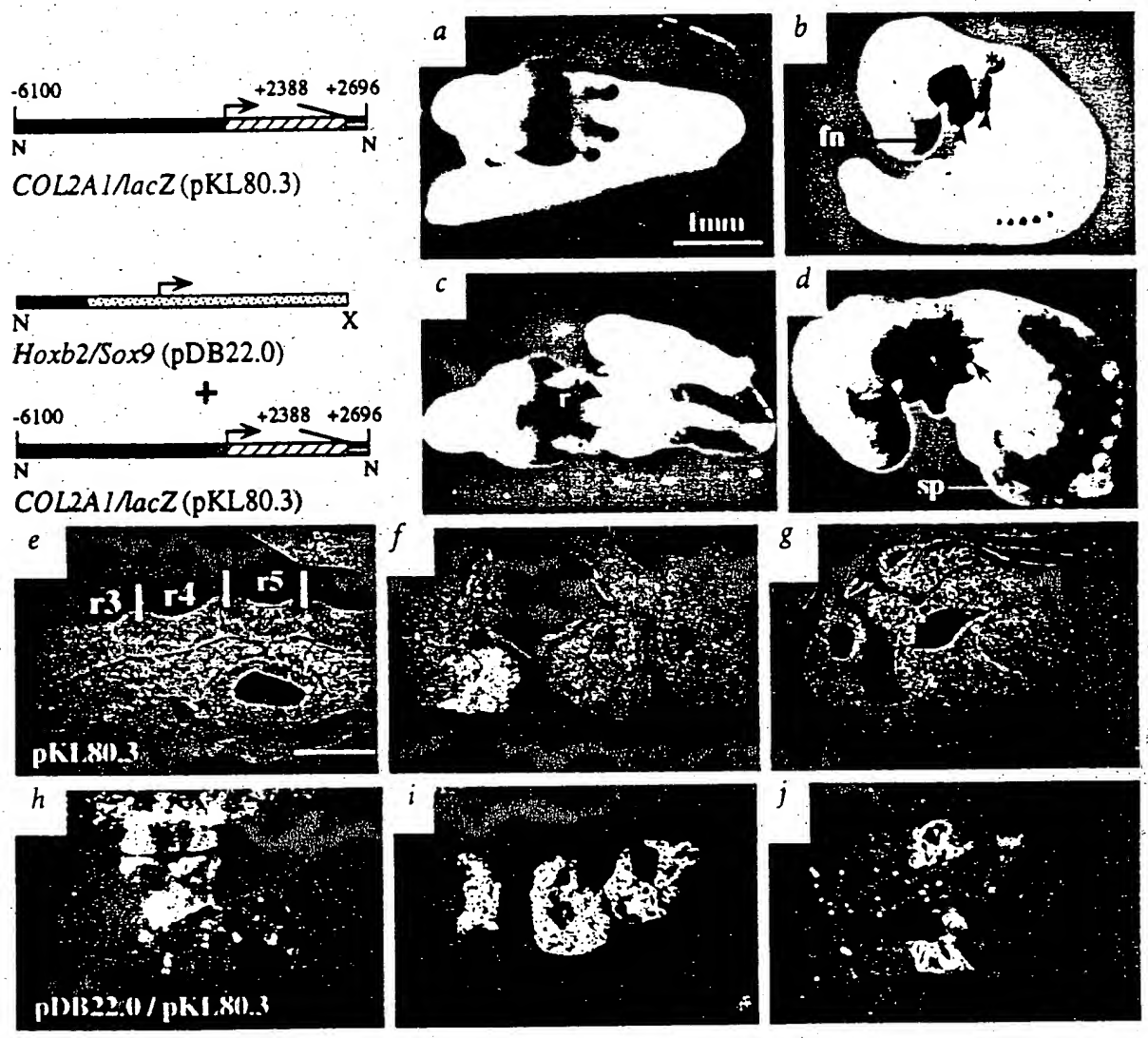

Fig. 3 *Trans*-activation of the *COL2A1-lacZ* reporter by SOX9 in transgenic mice. *a,b*, *COL2A1-lacZ* expression revealed by magenta-gal staining (see Methods) in *COL2A1-lacZ* (pKL80.3) transgenic embryo. The reporter is expressed in the frontonasal mesenchyme (fn), the mesenchyme of the first and second branchial arches (arrowheads), the otic vesicle (*) and the lower thoracic somites. *c,d*, Activation of *COL2A1-lacZ* expression in ectopic sites of the double (*COL2A1-lacZ* and *Hoxb2-Sox9*) transgenic embryo. The reporter is expressed in tissues originally expressing the pKL80.3 construct with additional expression in the third bronchial arch (arrow), rhombomeres (r) 3–5, the cervical and upper thoracic somites and the somatopleure (sp) (compare Figs 3a, b). *e–j*, A comparison of the *COL2A1-lacZ* expression in *COL2A1-lacZ* (pKL80.3, *e–g*), and *COL2A1-lacZ* (pKL80.3)/*Hoxb2-Sox9* (pDB22.0, *h–j*) embryos. In the double transgenic embryos, expression of the reporter is activated in the rhombomere (*h*), the third branchial arch (*i*) and the lateral plate and intermediate (urogenital) mesoderm (*j*). No *lacZ* expression is found in these tissues in the pKL80.3 transgenic embryos. Boxes in line drawings are as for Fig. 2 except stippled boxes represent *Sox9* genomic DNA. Bent arrow shows position of translation start site. Bar for *e–j* = 100 μm.

different effects of C1M and C2M on transgene expression may reflect differing affinities of the sites for SOX9 or an impact of fragment length on binding abilities of factors.

Our results indicate that both binding sites are important for chondrogenic expression of the *COL2A1-lacZ* reporter in transgenic mice. These findings contrast with other reports showing that tandem copies of a 73-bp fragment containing the *Col2a1* equivalent of COL2C1 could not direct chondrocyte expression of a *lacZ* reporter gene in transgenic mice[6] while 12 copies of COL2C2 could[7]. These discrepancies may reflect the inability of short fragments to exert full regulatory activity *in vivo*.

To test whether SOX9 can *trans*-activate *COL2A1/Col2a1 in vivo*, we studied the effect of expressing SOX9 ectopically in pKL80.3 transgenic mouse embryos using a 2-kb enhancer element upstream of the *Hoxb2* promoter[15]. This enhancer element directs expression of a promoterless *lacZ* gene to the hindbrain (rhombomeres r3, 4 and 5), neural tube, lateral mesoderm, somites, and branchial arches 2 and 3 in 22 of 25 transgenic 9.5-day embryos (construct pDB28.0; Table 1).

This *Hoxb2* element was used to direct expression of *Sox9* (construct pDB22.0, Fig. 3) in double transgenic mouse embryos produced by pronuclear injection of pDB22.0 into pKL80.3 *COL2A1-lacZ* transgenic oocytes. In 21 of the 24 9.5-day double transgenic embryos, novel *lacZ* reporter expression was found in the hindbrain; third branchial arch and the lateral mesoderm (compare Fig.3*a,b,e–g* with *c,d, h–j*; Table 1). Ectopic expression of the reporter was also found in the dermamyotome, dorsal somatopleure (Fig. 3*j*) and the intermediate (nephrogenic) mesoderm that gives rise to the nephrogenic cords and the mesenchyme of the urogenital ridges. Expression at these sites was never seen in the parental transgenic pKL80.3 line (Fig. 3*a,b, e–g*), suggesting that SOX9 trans-activates the reporter specifically via the *COL2A1* sequences.

The activation of the reporter gene at the ectopic sites coincided with the expression of endogenous *Sox9*. *In situ* hybridization showed elevated levels of *Sox9* transcripts in r3, 4 and 5 (Fig. 4*b, c* compare with Fig. 4*a*), the third branchial arch and the lateral body wall mesoderm (Fig. 4*f*). In the lateral mesoderm, *Sox9* mRNAs co-localized

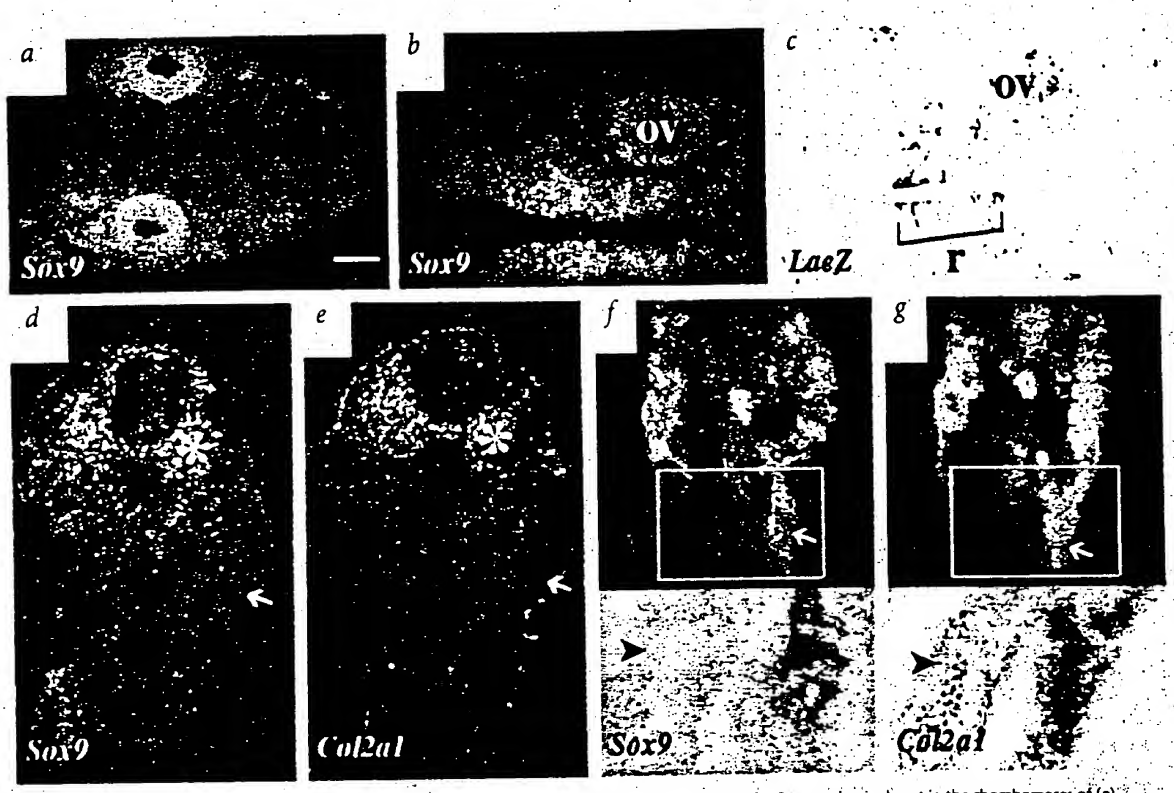

Fig. 4 Ectopic expression of *Sox9* and upregulation of endogenous *Col2a1*. *In situ* hybridization showing *Sox9* expression is absent in the rhombomeres of (*a*) *COL2A1-lacZ* (pKL80.3) transgenic embryo but ectopic expression of (*b*) *Sox9* and (*c*) the *lacZ* reporter is observed in the rhombomeres (bracket) of the *COL2A1-lacZ* (pKL80.3)/*Hoxb2-Sox9* (pDB22.0) embryos. In *COL2A1-lacZ* (pKL80.3) transgenic embryos, expression of *Sox9* (*d*) and *Col2a1* (*e*) is not detected in the lateral plate mesoderm. In contrast, ectopic expression of *Sox9* (*f*) and *Col2a1* (*g*) is clearly seen in the lateral plate mesoderm of double (pDB22.0/pKL 80.3) transgenic embryos (compare sites indicated with the arrow in *d–f*). The asterisk (*) marks normal endogenous expression of *Sox9* and *Col2a1* in the sclerotome. Insets (*f,g*) are bright-field pictures showing co-localization of *Sox9* (*f*) and *Col2a1* (*g*) transcripts with the *lacZ* reporter (magenta) in the same tissues in the lateral body wall. Although *Sox9* is normally expressed in the nephrogenic mesoderm[14], *Col2a1* expression is not observed[29]. Arrowheads (*f,g*) show the vitelline vein which acts as an internal control as the tissue is negative for both X-gal staining and *in situ* hybridization signal. Hybridization with sense control riboprobes show no significant signal above background (data not shown). In the double transgenic embryos the *COL2A1-lacZ* reporter gene was *trans*-activated in the nephrogenic mesoderm (not shown). It is possible that in the urogenital mesenchyme, endogenous levels of SOX9 in the pKL80.3 embryos were insufficient to activate the *COL2A1-lacZ* reporter gene. Levels of SOX9 above the threshold could account for *trans*-activation of the *COL2A1-lacZ* reporter in these tissues in the double transgenic embryos. Bar = 100 μm.

with novel expression of the endogenous *Col2a1* mRNA (Fig. 4*f,g*) suggesting that *Sox9 trans*-activated both the *COL2A1-lacZ* transgene and the endogenous type-II collagen gene at this site. Neither *Sox9* nor *Col2a1* was expressed at detectable levels in the third branchial arch or the lateral mesoderm in pKL80.3 and non-transgenic embryos (Table 1; Fig. 4*d,e*). Variability in *trans*-activation was found—for example, endogenous *Col2a1* was not upregulated in the hindbrain. This variability could be due to position effects causing low expression of SOX9 in the rhombomeres, competition by other SOX factors or a requirement for cooperating factors. The data are also consistent with the hypothesis that the biological activity of SOX9 is dosage dependant[3].

Regulatory links between a transcription factor and its downstream target are often inferred from co-transfection assays in cultured cells. This approach was used to demonstrate that the δ1-crystallin and *FGF4* genes are regulated by SOX2[16,17] and this is the only SOX protein for which downstream target genes have been identified. While such studies are valuable, it is important to establish that the regulatory relationship is relevant *in vivo*. For example, although KROX-20 could *trans*-activate the *Hoxa4* gene in co-transfection assays, the two genes are not co-expressed in the same tissues, arguing against direct interactions between them *in vivo*[18]. Evidence for transcription factor/target relationships *in vivo* is available for only a limited number of genes, such as KROX-20/*Hoxb2* and KROX-20/*Hoxa2*[15,19]. In this paper we provide strong evidence that *COL2A1* is a direct regulatory target of SOX9 in prechondrogenic and chondrogenic tissues *in vivo* suggesting that SOX9 binding mediates chondrogenic expression of *COL2A1*. As SOX9 can bind to sequences in the *COL2A1* first intron and mutation of the motifs abolished both SOX9 binding *in vitro* and cartilage-specific reporter gene expression in transgenic mice, it is likely that SOX9 acts via these sites. Definitive demonstration that these are the *in vivo* SOX9 binding sites will require generating transgenic mice simultaneously transgenic for *COL2A1-lacZ*, *COL2A1* sequences containing C1M or C2M linked to a different reporter and *Hoxb2-SOX9*.

It is interesting that the recently reported POU domain protein-binding site within the *Col2a1* first intron enhancer element[7] overlaps with the COL2C2 sequence. The differential expression of *Sox9* and *Col2a1* in non-chondrogenic tissues[4], the inappropriate transgene expression obtained when COL2C2 was mutated and the requirement for other tissue-specific co-factors for transcriptional activation by other HMG proteins such as LEF-1 and SOX2[16,17,20,21], suggest that SOX9 may not be solely responsible for mediating tissue-specific *COL2A1/Col2a1* expression.

SRY, LEF-1 and SOX2 are thought to be architectural factors which facilitate transcription via DNA bending[21–23]. Apart from the SOX9 binding motifs in the *COL2A1* enhancer there is also a perfect SRY consensus motif in the 5' flanking region of the gene. Cooperation between the first intron and the promoter has been shown to be important for activation of the rat *Col2a1* gene in chondrocytes[24] and for chondrocyte-specific expression of *COL2A1-lacZ* reporters in transgenic mice (KKHL, PPLT, KSEC unpublished). It is there-

fore possible that SOX9 facilitates interaction of factors bound at the promoter and the first intron partly by DNA bending.

The pathogenesis of the CD phenotype has been attributed to the haploinsufficiency of SOX9 (ref. 3). Our finding that the *COL2A1-lacZ* transgene can be *trans*-activated by SOX9 offers insight into the cause of skeletal malformation in CD associated with *SOX9* mutations. In humans and mice, a total loss of type-II collagen function in null mutants results in severe skeletal abnormalities but individuals heterozygous null for *COL2A1/Col2a1* are relatively mildly affected[25–27]. Therefore, the partial loss of type-II collagen in the context of SOX9 haploinsufficiency is unlikely to be the sole cause of CD abnormalities. Instead, the partial loss of SOX9 activity may lead to dysregulation of target gene(s) such as *COL2A1* as well as other downstream genes encoding different types of cartilage matrix components. Further investigation of the molecular mechanisms underlying CD should now focus on the role of SOX9 in the transcriptional regulation of other genes that are critical to chondrogenesis and bone formation.

Methods

Plasmids and gene constructs. The *COL2A1-lacZ* reporter pKL80.3 contained 6.1 kb 5' flanking DNA cloned upstream of a promoterless *E. coli lacZ* reporter gene cassette containing the ATG and polyA signal and 309 bp of the first intron (+2388 to +2696) cloned downstream of the reporter gene in pPolyIII I as shown in Fig. 2. Both pKL39 and pKL43 are similar to pKL80.3 except that they contain the C1M mutation at +2456 to +2461 (pKL39) or C2M mutation +2402 to +2407 (pKL43, see section on EMSA).

The ectopic expression construct (pDB28.0) contained 2 kb of the *Hoxb2* enhancer element[15] fused upstream of the promoterless *lacZ* gene cassette in pKS. The ectopic expression construct pDB22.0 was produced by ligating the 2-kb *Hoxb2* fragment upstream of a 7.5-kb *Eco*RI genomic fragment containing the *Sox9* gene isolated from a 129Sv library in lambda fix in pBS KSII– (Stratagene).

Electrophoretic mobility shift assays. (EMSA). EMSA using GALSOX9 fusion protein expressed in COS-1 cells was performed as described[4]. For competition analysis, 10- (10 ng) or 100-fold (100 ng) excess of end-filled, unlabelled oligonucleotide were included in the initial incubation. Sequences of the sense strand of double-stranded oligonucleotides used (probes and competitor DNAs) were:

SoCM 5'–GATCAGACTGAGAACAAAGCGCTCTCACACGATC–3';
SRYC5'–GATCCGGACTAATAAACAATAAAGTCGACGGATC–3';
COL2C1,5'–GATCCCCCTCTCCCACAATGCCCCCCTGTGGATC–3';
5'C1M,5'–GATCCCCCTCTCCAAGCCAGCCCCCCTGTGGATC–3';
COL2C2,5'–GATCCCTCGAGAAAAGCCCCATTCATGAGAGGATC–3';
C2M, 5'–GATCCCTCGAGAAAAGCCCCGCCGGCGAGAGGATC–3';

with the SOX binding motifs and mutations thereof shown in bold.

Transgenic mice. Single transgenic mice were produced by pronuclear injection of the various constructs into CBA/C57BL6 F1 oocytes. Double transgenic mice were produced by injection of pDB22.0 into oocytes heterozygous for the pKL80.3 construct from the mouse line KL18. Transgenic mice were screened by PCR and/or Southern analysis.

X-galactose staining and histology. Embryos were collected and fixed briefly in 4% paraformaldehyde in PBS before X-gal staining was performed essentially as described[28]. Colour development was carried out using either conventional X-gal, which yields a blue colour, or Magenta-gal (BioSynth). Colour development times varied from 2–6 h, depending on whether the embryos were processed for RNA *in situ* hybridization. Embryos were then post fixed in 4% paraformaldehyde in PBS overnight, dehydrated and embedded in paraffin wax. Sections of magenta-gal stained embryos were counterstained with nuclear fast red for histological analyses.

In situ hybridization assays. Mouse embryos were collected and processed for *in situ* hybridization as described[4]. Single-stranded, ^{35}S-labelled sense and antisense riboprobes were generated from subclones containing mouse *Col2a1* and *Sox9* gene exons as described[4,29]. K5 photographic emulsion (Ilford) was used for autoradiography and toluidine blue was used for counterstaining. Photomicrographs of sections were taken using Kodak Ektachrome 64 ASA film on a Zeiss Axiophot microscope under bright-field or dark-field illumination.

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